PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Matilde **BUSTOS DE ABAJO,** et al.

Serial No.: 10/798,219 Group No. 1633

Filed: March 11, 2004 Examiner: A.M.S. Wehbe

Confirmation No.: 3487

For: USE OF CARDIOTROPHIN IN LIVER DISEASES

Attorney Docket No.: **U 015070-8**

Commissioner for
Patents P. O. Box 1450
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DECLARATION UNDER 37 CFR 1.132

I, Matilde BUSTOS DE ABAJO, declare and say as follows:

1. I am a co-inventor of the invention described and claimed in US Patent Application Serial No. 10/798,219 ("the application"). I make this declaration in support of the application. My curriculum vitae is annexed hereto as Exhibit 1.

2. I believe that, as of the application filing date, a person of skill in the art to which the application pertains would have had an advanced degree in hepatology or the like and/or at least 5 years of experience working in this area. Such person would have knowledge of the publications discussed below.

3. The invention described and claimed in the application is based at least in part upon our discovery that the administration of cardiotrophin-1 (CT-1) to a subject whose liver has suffered injury (as occurs, e.g., when functional liver mass is diminished or the liver

is resected) can provide a therapeutic effect to the subject. In particular, we found, unexpectedly, that CT-1 not only prevents liver damage in healthy cells but it has also anti-apoptotic activity in already injured livers resulting in a marked therapeutic effect.

4. To explain, in an injured liver, several hepatocyte populations might co-exist, depending on its level of damage: dead hepatocytes, damaged hepatocytes and non-affected (healthy) hepatocytes. We found that CT-1 has regenerative activity in such injured livers inducing proliferation of surviving hepatocytes that can replace the dead ones. Equally and additionally crucial, CT-1 has antiapoptotic activity that prevents death of injured hepatocytes and healthy hepatocytes in the presence of an agent toxic for the liver. This cytoprotective activity increases the pool of surviving hepatocytes when the liver is injured. This facilitates the regenerative response of hepatocytes that will restore the liver functional mass.

5. I understand that the examiner of the application has cited the following publications to show that, as of the filing date of the application, one of skill in the art would have had a reasonable expectation of success in the use of CT-1 to treat a subject with an injured liver that has suffered a functional loss of liver cells: Jin et al, (1996) Cytokine, Vol. 8 (12) 920-926; Costa et al, U.S. Patent Application Publication 2002/0187936 and Hogaboam et al, U.S. Patent 6,719,969. In particular, I understand that the Examiner contends that: (a) Jin et al's disclosure that CT-1 induces liver growth *in vivo* demonstrates that CT-1 can stimulate hepatocyte proliferation and/or differentiation; and (b) the disclosure in Costa and Hogaboam that **different** proteins (i.e., proteins other than CT-1) that induce hepatocyte proliferation may be useful to treat subjects with liver damage provides a reasonable expectation that CT-1 would also be useful to treat subjects with liver damage. I respectfully disagree with these contentions.

6. With respect to the examiner's contention (a), Jin et al. do not provide any data or evidence showing hepatocyte replication or proliferation after CT-1 administration, but just an increase of the liver weight. No further comments or suggestions about the meaning of such observation are provided. It should be considered that effects other than hepatocyte proliferation can account for the increase in liver weight in the mice in the Jin et al study. Thus, one of skill in the art could not have concluded that the increased liver

weight in the Jin et al. study was due to hepatocyte proliferation. For example, hepatomegaly (that is increased liver weight) can be observed in several situations such as steatosis, toxic hepatitis, cholestasis, acute liver failure, congestive heart failure (right ventricular failure). On the other hand, different factors can increase the weight of healthy livers by increasing the size of liver cells without increasing the number of hepatocytes (that is, without stimulating the replication of liver cells). For instance, dexamethasone is able to induce hypertrophy of the hepatocytes increasing the liver weight; while this drug induces a severe inhibition of DNA synthesis. In this case, liver hypertrophy is transient, because after dexametaxone withdrawal, hepatocyte size is re-established within a few days. (Nagy P, Teramoto T, Factor VM, Sánchez A, Schnur J, Paku S, Thorgeirsson SS. Reconstitution of liver mass via cellular hypertrophy in the rat. *Hepatology,* 2001 Feb; 33(2):339-345).

7. Indeed, experimentation that I performed or that was performed under my supervision and control shows that the increased weight described in Jin et al was likely **not** due to such proliferation. Specifically, we have administered chronic doses of CT-1 to healthy mice, as Jin et al. have done (Jin H, Yang R, Keller GA, Ryan A, Ko A, Finkle D, Swanson TA, Li W, Pennica D, Wood WAI, Panoni NF. In vivo effects of cardiotrophin-1. *Cytokine.* 1996; 8 (12): 920-926) to confirm data by these authors and to investigate the mechanism of increased liver weight when CT-1 was given to mice with normal livers. As controls we used mice given saline (vehicle) instead of CT-1. At the end of the experiment, animals were sacrificed and the livers were analyzed. Liver weight was increased in rCT-1-treated animals compared to saline-treated mice (1.112 ± 0.02 *versus* 0.977 ± 0.03). The histopathological study of the liver of CT-1-treated mice showed no mitosis and the immunohistochemistry study for Ki-67 was negative (Figure 1A). Ki67 is a nuclear protein that is expressed in proliferating cells. Ki-67 has been used as a marker for cell proliferation. Furthermore, the expression of cyclin D1 in the livers from rCT-1 treated animals was negative as well as in the saline-treated animals (Figure 1B). The expression of cyclin D1 in the liver regulates transition from G1 to S phase in cell cycle and is used as a marker for cell proliferation.

The Figure 1 clearly shows that CT-1 treatment to healthy mouse does not induce hepatocyte proliferation.

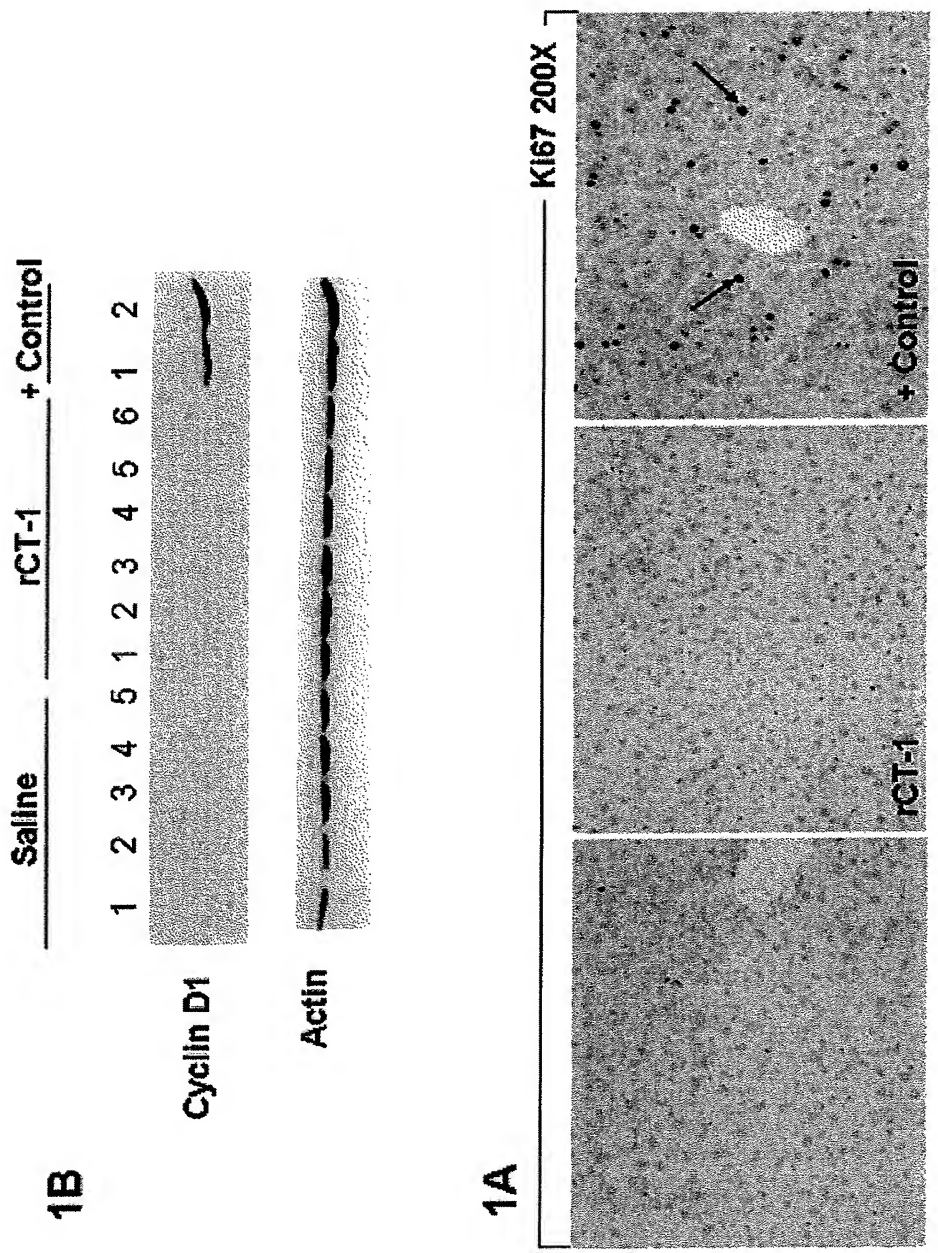

Figure 1:

Saline: Livers from saline-treated animals

rCT-1: Livers from rCT-1-treated animals

Positive control (+ Control): To show that the technique is reliable we employed a positive samples obtained from mouse livers after partial hepatectomy (proliferation of hepatocytes)

The above data indicate that, in spite of the increased liver weight, we could not see any hepatocyte replication. I accordingly believe that the increased liver weight observed by chronic CT-1 treatment is not due to hepatocyte replication.

8. With respect to the examiner's contention (b), I respectfully call attention to other publications that show why one of skill in the art would not expect that any and all substances with hepatocyte proliferative effects could be effectively administered to patients suffering from liver damage. By way of example, Bockhorn et al. (Bockhorn M, Schóllmann S, Optiz B, Sotiropoulos GC, Sheu SY, Niehaus E, trippler M, Frilling A, Broelsch CE, Schlaak JF. Vascular endothelial growth factor does not improve liver regeneration and survival after 90% subtotal liver resection. *Hepatol Res.* 2007 May;37(5):353-9) show that, although VEGF is a well known growth factor, the administration does not improve liver regeneration and survival after 90% subtotal liver resection. As another example, Klemm et al. (Klemm K, Eipel C, Cantré D, Abshagen K, Menger MD, Vollmar B. Múltiple doses of erythtropoietin impair liver regeneration by increasing TNF-alpha, the Bax to Bcl-xL ratio and apoptotic cell death. *PloS One* 2008;3(12):e3924. Epub 2008 Dec 11.) observe that, although erythroopoietin has been recognized as an antiapoptotic, mitogenic and tissue-protective cytokine, múltiple doses of erythropoietin impaired liver regeneration by increasing apoptotic cell death (Klemm K, Eipel C, Cantré D, Abshagen K, Menger MD, Vollmar B. Múltiple doses of erythtropoietin impair liver regeneration by increasing TNF-alpha, the Bax to Bcl-xL ratio and apoptotic cell death. *PloS One* 2008;3(12):e3924. Epub 2008 Dec 11).

9. In view of the above considerations, I respectfully submit that the publications cited by the examiner would not have provided one of skill in the art with even a reasonable expectation of success in the use of CT-1 for the treatment of liver damage in a subject.

10. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity

of the application or any patent issued thereon.

Date: 09 / 09 / 2009

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Curriculum Vitae Matilde Bustos

Personal

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Education

1981/1982-1986/87. School of Medicine. University of Granada (Spain). August 1987.

1987/1988-1988-1989. PhD program in Pathology. University of Granada (Spain).

1988-1992. MD/PhD program in Pathology. Fellow in Pathology (resident fellow). Department of Pathology. Hospital Universitario. Granada. (Spain).

July 1992. Ph.D. earned *cum laude* on July-1992 under the direction of Prof. M. Gómez-Morales and Dr. R. Garcia del Moral. Dissertation title: "leucocytes sub-populations and Cyclosporin-A deposits in transplant kidney biopsies"

October 1993-April 1997. Post-doctoral fellow at Duke University (North Carolina, USA). Transplant Laboratory, under the direction of Dr. Jeffrey L. Platt. Allo- and Xeno-transplantation.

November 1997-current. Research Investigator at Center for Applied Medical Research (CIMA). University of Navarra. Pamplona (Spain). In the Area of Hepatology and Gene Therapy

Research Experience

During post-doctoral research:

October 1993- April 1997

- Fellowship from the Ministery of Health.(FIS). October 1993—October 1994. Transplant laboratory. Project of Xeno and allo-transplants. Director Dr. Jeffrey L. Platt.

- Fellowship from the Ministery of Education and Science (MEC). November 1994- November 1996. Xeno and allo-transplantation
- Research Associate at Duke University. December 1996- April 1997. Xeno and allo-transplantation

November 1997- Current
-Department of Hepatology and Gene therapy. Liver damage and liver regeneration. Hepatoprotection. Cytokines and growth factors. IL-6 family of cytokines: IL-6, cardiotrophin-1, CNTF, LIF and oncostatin M. Metabolism.

Awards/Grants

1993: Prize in the first Hispano-American Meetting of Nephrology. Barcelona 1993.

2001: 2nd Prize in Falk Symposium. Hepatocyte Transplantation. Hannover. October 2001.

2000: Grant: "Role of cardiotrophin-1 in liver regeneration. Mechanism and gene therapy with the adenovirus-cardiotrophin-1 in liver diseases". Ref# 96/2000. Gobierno de Navarra. Principal Investigator: Matilde Bustos.

2000/2001 Grant: "Identification of growth and differentiation factors of hepatic progenitors and stem cells from bone marrow involved in the liver regeneration". Ref# 01/0723. Ministery of Health. Principal Investigator: Matilde Bustos.

2004: Grant "Cardiotrophin-1 knock-out mice for the study the role of this cytokine in the liver development and the experimental liver damage". Ref # 17/2004 Gobierno de Navarra. Principal Investigador: Matilde Bustos.

2004: Grant "Hepatoprotective role of cardiotrophin-1. Study of the knock-out mice for cardiotrophin-1 in the acute and chronic liver damage and liver regeneration. Ministery of Health (FIS) (Ref# PI041321). Principal Investigator: Matilde Bustos.

2006: Grant "Role of CT-1 in obesity and liver. Potential therapeutic role of CT-1 in these pathologies. Principal Investigator: Matilde Bustos.

2006 Grant " Role of Cardiotrophin-1 in NASH and associated co-morbidities. PI070698. Principal Investigator: Matilde Bustos.

List of Publications

Gómez-Morales M, Muñoz M, **Bustos M**, Escobar F. Diffuse sclerosing papillary carcinoma of the thyroid. *Clinical Endocrinology* 1991;34:432.

Nogales FF, Beltran E, Pavcovich M, **Bustos M**. Ectopic somatic endoderm in secondary human yolk sac. Human Pathology. 1992;23:921-924.

Lardelli P, Aguilar D, Gómez-Morales M, Anton I, Navarro N, Montes A, **Bustos M**, Garcia del Moral R. Presence of cytomegalovirus genome and leukocyte subsets in renal transplant biopsies. Relationship with prognosis. *Pathology Research and Practice*. 1994; 190(2):142-150.

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Parker W, Saadi S, Lin S, **Bustos M**, Platt JL. Transplantation of discordant xenografts. *Immunology Today*. 1996;17(8):373-378.

Bustos M, Platt JL Molecular bases in xenograft. *Molecular Diagnosis* 1996;1(3): 225-233.

Bustos M, Platt JL Platelet-endothelial cell interactions in a xenograft model. *Transplantation Proc.* 1997;29:886.

Bustos M, Coffman TM, Saadi S, Platt JL Modulation of eicosanoid metabolism in endothelial cells in a xenograft model: Role of cyclooxygenase-2. *J Clin Invest* 1997; 100(5): 1150-1158

Parker W, Holzknecht ZE, Song A, **Bustos M**, Reissner JK, Everett ML, Platt JL. Fate of antigen in xenotransplantation: implications for acute vascular rejection and accomodation. *Am J Pathol.* 1998 153 (3): 829-839.

Mannon RB, Kopp JB, Ruiz P, Griffiths R, **Bustos M,** Platt JL, Coffman TM. Chronic rejection of mouse kidney allografts. *Kidney* Int 1999; 55 (5):1935-1944.

Mannon RB, Doyle C, Griffiths R, **Bustos M,** Platt JL, Coffman TM. Altered intragraft immune responses and improved renal function in MHC class II deficient mouse kidney allografts. *Transplantation* 2000 May 27;69(10):2137-43.

Melero I, Duarte M, Ruiz J, Sangro B Galofre JC, Mazzolini G, **Bustos M,** Qian C, Prieto J. Intratumoral injection of bone-marrow derived dendritic cells engineered to produce interlekin-12 induces complete regression of established murine transplantable colon adenocarcinomas. *Gene Therapy* 1999; 6:1779-1784.

Torres L, Garcia-Trevijano ER, Rodriguez JA, Carretero V, **Bustos M**, Mato JM, Avila M. Induction of TIMP-1 expression in rat hepatic stellate cells and hepatocytes: a new role for homocysteine in liver fibrosis. *Biochimica et Biophysica Acta* 1999; 1455:12-22.

Holzknecht ZE, Coombes S, Blocher BA, Plummer TB, **Bustos M**, Lau CL, Davis RD, Platt JL. Evidence of immunocomplex formation in pulmonary xenografts. *Transplant Proc.* 2000 Aug;32(5):1141.

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Mazzolini G, Narvaiza I, **Bustos M,** Duarte M, Tirapu I, Bilbao R, Qian C, Prieto J, Melero I. αvβ3 integrin-mediated adenoviral transfer of interleukin-12 at the periphery of hepatic colon cancer metastases induces VCAM-1 expression and T-cell recruitment. *Molecular Therapy* 2001; 3(5):1-8.

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Beraza N, Marqués JM , Martínez-Ansó E, Iñiguez M, Prieto J, **Bustos M.** Interplay among cardiotrophin-1, prostaglandins and vascular endothelial growth factor in rat liver regeneration. *Hepatology* 2005 March 41 (3): 460-469

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Iñiguez M, Berasain C, Martinez-Ansó E, **Bustos M**, Fortes P, Pennica D, Avila MA, Prieto J. Cardiotrophin-1 defends the liver against ichemia-reperfusion injury and mediates the protective effect of ischemic preconditioning. *J Exp Med* 2006 Dec 25;203(13):2809-15.

Marques JM, Belza I, Holtmann B, Pennica D, Prieto J, **Bustos M.** Cardiotrophin-1 is a natural defense against apoptosis. *Hepatology* 2007 Feb 26;45(3):639-648.

Domínguez-Soto A, Aragoneses-Fenoll L, Gómez-Aguado F, Corcuera MT, Clária J, García-Monzón C, **Bustos M**, Corbí AL. The pathogen receptor liver and lymph node sinusoidal endotelial cell C-type lectin is expressed in human Kupffer cells and regulated by PU.1. *Hepatology* 2009 Jan;49(1):287-96

Lorente-Cebrian S, **Bustos M**, Martí A, Martinez JA, Moreno-Aliaga MJ. Eicosapentaenoic acid stimulates AMP-activated protein kinase and increases visfatin secretion in cultured murine adipocytes. Clin Sci (Lond). 2009 Aug 14;117(6):243-9.

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Participations in Symposiums

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Meetings

Meeting of United States and Canadian Academy of Pathology. Toronto 1994. "Synthesis of endothelin-1 and prostagladins by porcine endothelial cells as a potential mechanism underlay vasoconstriction in xenotransplantation" **Bustos M** and Platt JL.

III International Congress for Xenotransplantation. Boston 1995 "Modulation of endothelial cells by xenogenic serum and platelets" **Bustos M** and Platt JL.

28th Annual Meeting American Society of Nephrology. San Diego 1995. "Elaboration of thromboxane A2 and endothelin-1 by endothelial cells in response to antibodies, complement and platelets" **Bustos** M, Saadi S, Platt JL.

International Meeting of transplantation. Barcelona. 1996 "Platelet-endothelial cell interactions in a xenograft model" **Bustos M**, Platt JL.

29th Annual Meeting of American Society of Nephrology. New Orleans 1996. "Release of eicosanoids and expression of cyclooxygenase-2 in a xenograft model" **Bustos M**, Coffman TM, Platt JL.

15th Annual Scientific Meeting for the American Society of Transpant Physicians. Chicago 1997 "Chronic rejection of mouse kidney allografts: The role of direct allorecognotion" Mannon RB, Ruiz P, **Bustos M**, Griffiths R, Platt JL, Coffman TM. "Allograft rejection in kidneys lacking normal MHC expression" Mannon RB, Ferri K, **Bustos M**, Ruiz P, Platt JL, Coffman TM

34th Meeting of the European Association for the Study of the Liver. Naples. April 1999. "Toxicity study after intraportal administration of the adenovirus-thymidin kinase gene and gancyclovir" **Bustos M,** Sangro B, Alzuguren P, Gil A, Herraiz M, Ruiz J, Bilbao R, Qian C, Prieto J. "Effective and safe gene therapy of experimental liver cancer (HCC) by intratumoral injection of a defective adenovirus containing the thymidine kinase (tk) gene" Sangro B, Bustos M, Barajas M, Herraiz M, Alzuguren P, Gil A, Ruiz J, Bilbao R, Qian C, Prieto J.

50th Meeting of the American Association for the Study of Liver Diseases. Dallas. November 1999. "Liver failure caused by suicide gene therapy is due to mitochondrial dysfunction without mitochondrial DNA depletion". Herraiz M, **Bustos M,** Beraza N, Gil A, Alzuguren P, Ruiz J, Prieto J.

XXV Congreso de la Asociacion Española para el Estudio del Hígado. Madrid. Febrero 2000. "Daño hepático en la terapia con genes suicidas: un modelo para la activación de células ovales" **Bustos M**, Sangro B, Alzuguren P, Gil A, Herraiz M, Qian C, Ruiz J, Prieto J. Gastroenterol y Hepatol 2000; 23:108

3rd Annual Meeting of the American Society of Gene Therapy. Denver. May 2000.
"Liver failure caused by suicide gene therapy is due to mitochondrial dysfuntion without mitochondrial DNA depletion" Herraiz M, Sangro B, Montoya J, Beraza N, Gil A Alzuguren P, Qian C, Ruiz J, **Bustos M**, Prieto J. "Safety issues of interleukin-12 gene therapy using adenoviral vectors" Sangro B, Gil A, Diaz L, Ruiz J, Córdoba M, Barajas M, Alzuguren P, **Bustos M**, Qian C, Prieto J.

54th Annual Meeting of the American Association for the Study of Liver Diseases. Boston 2003
"Contribution of Cardiotrophin-1 in the prostaglandins and VEGF response in the regenerating liver. N Beraza, P Alzuguren, J Prieto, M Bustos.
"Cardiotrophin-1 is a potent hepatoprotective factor which defends the liver against ischemia-reperfusion injury" M Iñiguez, E Martinez-Ansó, N Beraza, M Bustos, P Fortes, J Prieto.

56th Annual Meeting of the American Association for the Study of Liver Diseases. San Francisco 2005. Cardiotrophin-1 is an essential factor in the natural defence of the liver against Fas-induced cell death.